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Synthesis of calf prochymosin (prorennin) in *Escherichia coli*

(synthetic oligonucleotide/gene expression/industrial enzyme)

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ABSTRACT **A gene for calf prochymosin (prorennin) has been reconstructed from chemically synthesized oligodeoxyribonucleotides and cloned DNA copies of preprochymosin mRNA. This gene has been inserted into a bacterial expression plasmid containing the *Escherichia coli* tryptophan promoter and a bacterial ribosome binding site. Induction of transcription from the tryptophan promoter results in prochymosin synthesis at a level of up to 5% of total protein. The enzyme has been purified from bacteria by extraction with urea and chromatography on DEAE-cellulose and converted to enzymatically active chymosin by acidification and neutralization. Bacterially produced chymosin is as effective in clotting milk as the natural enzyme isolated from calf stomach.**

Chymosin (rennin) is an aspartyl proteinase found in the fourth stomach of the unweaned calf, where it is responsible for limited proteolysis of κ-casein in milk (1), resulting in clotting (2). Full-length cDNA copies of the mRNA for chymosin have recently been cloned and their sequences have been determined (3–5). The primary translation product of the mRNA is a precursor protein, preprochymosin, that has a 16-amino acid leader peptide. This signal sequence is removed to produce the zymogen prochymosin, which in turn is converted to active chymosin under the acid conditions of the stomach by removal of the 42-amino acid propeptide from the NH_2-terminus (6).

Recombinant plasmids containing the preprochymosin coding sequence provided a choice of genes for expression in *Escherichia coli*—i.e., preprochymosin, prochymosin, or chymosin. Preprochymosin and chymosin were ruled out for the following reasons. For preprochymosin, the *E. coli* cell would have to recognize the eukaryotic signal peptide and process it accurately to prochymosin, which would then be found in the periplasmic space. Despite the fact that *E. coli* processes the rat preproinsulin gene in this manner (7), it does not, apparently, process preinterferon-α (8), preinterferon-β (9), or pregrowth hormone (10) in the same way. Direct expression of the chymosin gene would be expected to lead, by analogy with human growth hormone (11), to production of chymosin containing an additional methionine residue at the NH_2 terminus ([Met]chymosin). Apart from possible deleterious effects of the production of active chymosin in *E. coli*, there was also the possibility that an NH_2-terminal methionine would alter the activity or tertiary structure of the enzyme. The expression of prochymosin, on the other hand, offered positive advantages. First, the 42-amino acid propeptide can be removed *in vitro* by acidification followed by incubation at pH 6 (12) and, assuming that a [Met]prochymosin could be cleaved in similar fashion, the product would be authentic chymosin rather than [Met]chymosin. Second, since preliminary experiments indicated that the majority of *E. coli* proteins (about 90%) precipitated under the acidic chymosin maturation conditions and remained insoluble on neutralization, it was possible that purification as well as activation could be achieved.

We describe here the construction of *E. coli* plasmids designed to express the prochymosin gene from the *trp* promoter and the isolation and conversion of this prochymosin to enzymatically active chymosin.

MATERIALS AND METHODS

Materials. DNase I, pepstatin A, and phenylmethylsulfonyl fluoride were obtained from Sigma. Calf prochymosin (M_r 40,431) and chymosin (M_r 35,612) were purified from stomachs from 1-day-old calves (1). Rabbit antiprochymosin antiserum was provided by the National Institute of Medical Research (Mill Hill, London). Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, polynucleotide kinase was from Bethesda Research Laboratories, and calf intestinal alkaline phosphatase and S1 nuclease were from Boehringer Mannheim. Restriction enzymes were used under conditions recommended by the supplier. Oligodeoxyribonucleotides R29 (5′-phosphate-G-A-T-C-C-G-A-G-T-G-A-T-T-T-C-A-G-C) and R30 (5′-hydroxy-G-C-T-G-A-A-A-T-C-A-C-T-C-G) were synthesized by the phosphotriester procedure (13) using preferred *E. coli* codons (14).

Bacterial Strains. *E. coli* K-12 strain HB101 (15) containing the plasmids pCT54 or pCT70 were grown at 37°C in M9 medium (16) supplemented with glucose, all amino acids except tryptophan (and methionine when labeling proteins with [^{35}S]-methionine), and 100 μg of carbenicillin per ml. Large quantities of cells were prepared by growth for 10 hr to an OD_{600} of 10 (1×10^9 cells per ml) and harvested by centrifugation or ultrafiltration.

Assay of Chymosin Milk-Clotting Activity. Prochymosin from a calf or bacterial source was converted to catalytically active chymosin by acidification/neutralization (activation) immediately prior to assay. One-milliliter samples were incubated at pH 2.0 for 15 min at room temperature and then the pH was adjusted to 6.3 and incubation was continued for 1 hr. Insoluble proteins were removed by centrifugation for 1 min in a bench centrifuge and samples of the supernatant were taken for assay. A simple microtiter plate assay was developed for the measurement of milk-clotting activity. The final assay mixture (100 μl per well) was 12% (wt/vol) dried skim milk/20 mM $CaCl_2$/25 mM phosphate buffer, pH 6.3, and chymosin was incubated at 37°C for 15 min. The plate was inverted and any unclotted milk was allowed to drain over absorbent paper prior to photography of the wells containing clotted milk. The assay was capable of detecting approximately 0.02 μg of chymosin although the sensitivity could be increased by prolonged incubation.

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Abbreviation: bp, base pair(s).

Purification of Chymosin from *E. coli*. Cells [100 g (wet weight)] were lysed with lysozyme/sodium deoxycholate (17) and treated with DNase (18). The resulting suspension was centrifuged at 10,000 × g for 30 min at 4°C, the pellet formed was suspended in 300 ml of 50 mM Tris·HCl, pH 8/0.1 M NaCl/1 mM EDTA and recentrifuged as above. Washed cell debris was dissolved in 200 ml of 9 M urea (freshly deionized)/50 mM Tris·HCl, pH 8/0.5 M NaCl/1 mM EDTA and dialyzed overnight at 4°C against 5 liters of 50 mM Tris·HCl, pH 8/0.5 M NaCl/1 mM EDTA/10% (vol/vol) glycerol. The suspension was centrifuged as above, and the supernatant diluted 1:4 with 0.01 M Tris·HCl, pH 8/1 mM EDTA/10% (vol/vol) glycerol and applied to a 200-ml column (4.7 × 15 cm) of DEAE-cellulose previously washed and equilibrated in 0.01 M Tris·HCl, pH 8/1 mM EDTA/10% (vol/vol) glycerol. Adsorbed proteins were eluted with a linear gradient of NaCl (0.05–0.5 M) in equilibration buffer. Prochymosin-containing fractions were detected by assaying activated samples for milk-clotting activity and by $NaDodSO_4$/polyacrylamide gel electrophoresis of fractions across the profile. The enzyme eluted reproducibly as a single peak at a NaCl concentration of 0.25 M. Appropriate prochymosin fractions with a purity at this stage of approximately 50% were pooled and activated as described above to generate chymosin. The insoluble protein impurities generated by acidification were removed by centrifugation and pure chymosin was stored at −20°C. Further details of the isolation of chymosin from *E. coli* will appear elsewhere.

RESULTS AND DISCUSSION

Construction of the Prochymosin Gene. The approach used for the bacterial synthesis of [Met]prochymosin was to reconstruct a gene *in vitro* from restriction fragments of a cDNA clone and then use synthetic DNA fragments for insertion into a bacterial plasmid immediately downstream from the strong *E. coli trp* promoter, a functional ribosome binding site and ATG. As shown in Fig. 1*A*, a *Bam*HI restriction site is conveniently located between codons 5 and 6 of the prochymosin sequence. Two oligodeoxyribonucleotides were designed to restore codons 1–6 and produce a blunt 5′ end with a 5′-OH group. These oligomers were hybridized and ligated to the 474-base-pair (bp) *Bam*HI fragment from plasmid 118 (3) and the resulting DNA was cleaved with *Eco*RI to produce a 478-bp DNA fragment coding for the 5′ end of prochymosin. The 3′ end of the prochymosin gene was isolated as an *Eco*RI/*Hin*dIII fragment (Fig. 1*B*) of ≈800 bp from plasmid C5. The *Hin*dIII cohesive end was dephosphorylated by calf intestine alkaline phosphatase during isolation of the fragment to prevent unwanted ligation

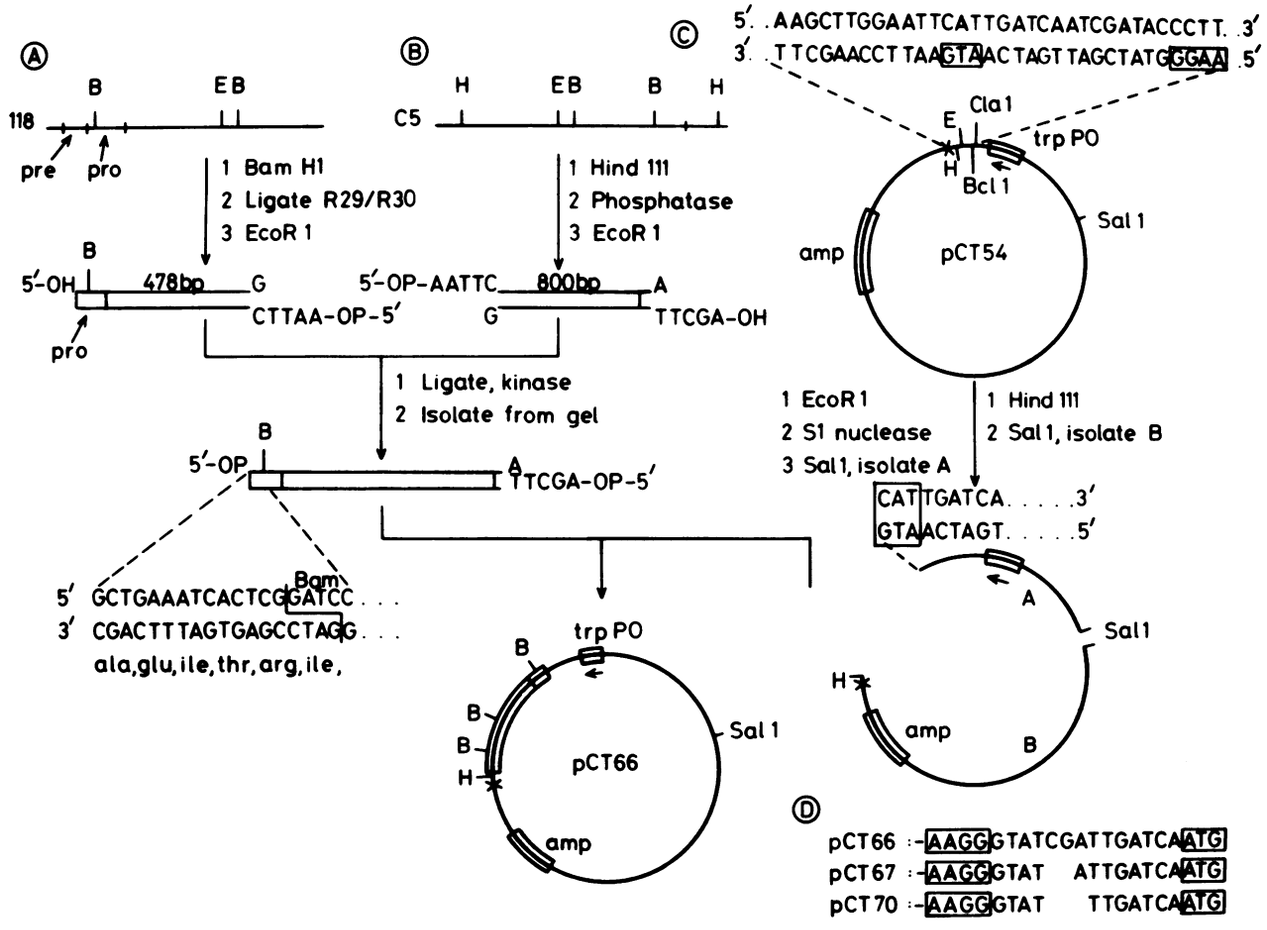

FIG. 1. Construction of plasmids for the direct synthesis of prochymosin in *E. coli*. Plasmid 118 (3) contains the calf preprochymosin gene inserted into the *Pst* I site of pAT153. Plasmid C5 contains the 3′ region of the calf preprochymosin gene recloned from plasmid A36 (3) into the *Hin*dIII site of pAT153 by using *Hin*dIII linkers. (*A*) Isolation of a 5′ fragment of the gene linked to synthetic oligonucleotides R29 and R30, which replace codons 1–5. This fragment contains an *Eco*RI cohesive 3′ end. (*B*) Isolation of a 3′ fragment of the gene as an *Eco*RI/*Hin*dIII fragment of ≈800 bp from plasmid C5. (*C*) Construction of the prochymosin expression vector by ligation of the reconstructed prochymosin gene to two fragments of pCT54 containing the bacterial gene expression control signals (fragment A) and a selective marker (β-lactamase, fragment B). (*D*) Digestion of pCT66 with *Cla* I and nuclease S1 to produce plasmids pCT67 and pCT70, which have a shorter Shine–Dalgarno ATG distance. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII. The boxed sequences AAGG and ATG represent the Shine–Dalgarno sequence of the *trp* leader and the initiation codon of prochymosin, respectively. The approximate location of the T7 transcription terminator (X) is shown on the plasmid vectors.

at this end in subsequent reactions. The 5′- and 3′-end fragments were recombined through the *Eco*RI cohesive ends and ligated by using T4 DNA ligase. The reconstructed prochymosin gene of 1,250 bp was isolated by gel electrophoresis after phosphorylation of the 5′ ends by using polynucleotide kinase.

Construction of the Prochymosin Expression Vector. The vector designed for the expression of prochymosin was pCT54 (Fig. 1*C*), which contains the *E. coli trp* promoter, operator, and leader ribosome binding site (19). In addition, 14 nucleotides downstream of the ribosome binding site is an initiator ATG followed immediately by *Eco*RI and *Hin*dIII sites and the terminator for *E. coli* RNA polymerase from bacteriophage T7 (20). This plasmid was digested with *Eco*RI and treated with nuclease S1 to generate a blunt end directly after the ATG; a fragment (A in Fig. 1*C*) containing the *trp* promoter and *trp* leader ribosome binding site was prepared by *Sal* I digestion and polyacrylamide gel electrophoresis. A second fragment (B in Fig. 1*C*) containing the β-lactamase gene was prepared from pCT54 by *Hin*dIII/*Sal* I digestion. The prochymosin gene was ligated to fragment A and fragment B (30 ng of prochymosin gene fragment/20 ng of vector fragment A/90 ng of vector fragment B) and the mixture was used to transform *E. coli* HB101 to ampicillin resistance (21). Twelve resistant clones were screened for the presence of a prochymosin gene by isolating plasmid DNA (22) from 1-ml overnight cultures grown in L broth (14) and digesting with *Bam*HI. The sequences of eight plasmids producing the predicted fragment pattern were determined from the *Cla* I site (23). All eight plasmids had the same sequence; that of pCT66 is shown in Fig. 1.

Expression of the Prochymosin Gene. *E. coli* cells containing pCT66 were tested for expression of prochymosin. Because transcription from the *trp* promoter is induced by lack of tryptophan in the medium, cells were grown in minimal medium to late exponential phase and total cellular extracts were prepared and subjected to electrophoresis on $NaDodSO_4$/poly-

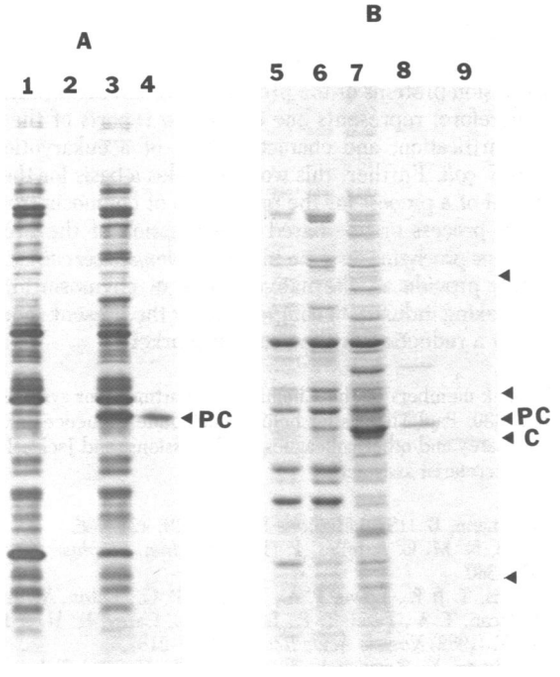

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis analysis of prochymosin expression in *E. coli*. (*A*) Immunoprecipitation of [^{35}S]methionine-labeled expressed prochymosin. Cells were grown to midlogarithmic phase and pulse labeled for 2 min with [^{35}S]methionine (70 μCi/ml; 1 Ci = 37 GBq). Labeled cells were washed with 50 mM Tris·HCl, pH 8.0/0.15 M NaCl and disrupted by boiling in the same buffer containing 1% (wt/vol) $NaDodSO_4$. Total cell proteins (in a volume of 100 μl) were incubated with excess rabbit antiprochymosin antiserum (10 μl of neat untreated serum) and specifically precipitating proteins were recovered (28). Total cell proteins and immunoprecipitates derived from cells containing plasmids pCT54 (lanes 1 and 2) and pCT70 (lanes 3 and 4) are shown after electrophoresis and autoradiography. (*B*) Accumulation, isolation, and activation of *E. coli* prochymosin. Proteins were stained with Coomassie blue. Lanes: 5, total proteins from *E. coli* containing pCT54 [washed cells (1.2×10^7) were boiled in 20 μl of sample buffer for 3 min and applied to the gel]; 6, total proteins from *E. coli* containing pCT70 prepared for electrophoresis as described above; 7, proteins from *E. coli* containing pCT70 were applied to the DEAE-cellulose column; 8, partially purified prochymosin after DEAE-cellulose chromatography—the proteins shown correspond to the peak clotting-activity fraction; 9, chymosin derived from the protein shown in lane 8 after activation and centrifugation. Authentic prochymosin (PC), chymosin (C), bovine serum albumin (M_r, 66,000), ovalbumin (M_r, 45,000), and pancreatic trypsinogen (M_r, 24,000) were used as markers and are indicated by arrowheads.

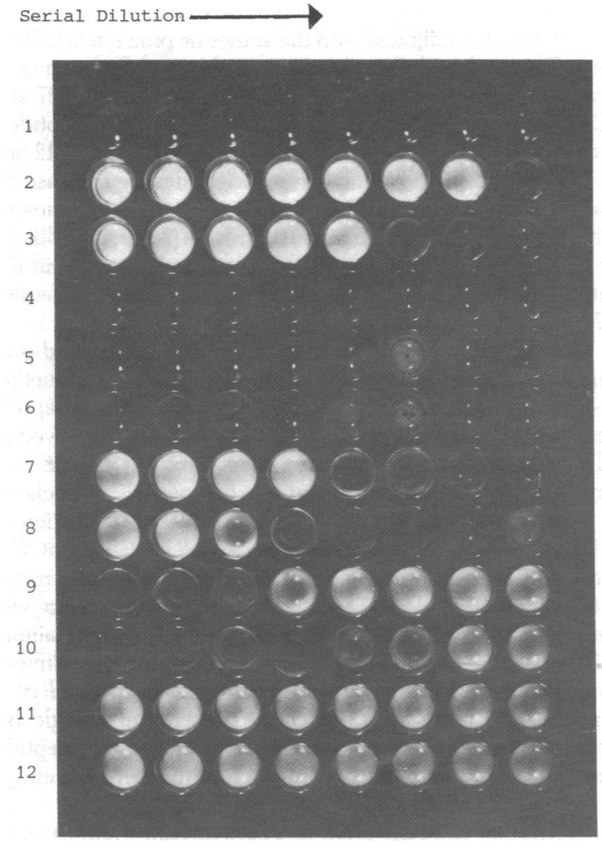

FIG. 3. Comparison of milk-clotting activity of chymosin obtained from calf with that obtained from *E. coli* containing pCT70. A microtiter plate showing clotted milk in the wells is shown. Stated concentrations of enzyme (and inhibitor, if added) were present in wells on the extreme left. Enzyme (and inhibitors) were serially diluted 1:1 in reaction mixture. Rows: 1, calf prochymosin at 18 μg/ml; 2 and 3, calf chymosin at 40 and 8 μg/ml, respectively. Rows 4–8: 25-μl samples of urea solubilized and dialyzed extracts of *E. coli* containing pCT54 or pCT70 before and after activation were assayed—4, pCT54-derived extract before activation; 5, as in row 4 but after activation; 6, pCT70-derived extract before activation; 7, a 1:4 dilution of the enzyme used in row 6 after activation; 8, a 1:12 dilution of the enzyme used in row 6 after activation. Rows 9–12: *E. coli*-derived chymosin purified by DEAE-cellulose chromatography was compared with calf chymosin for susceptibility to protease inhibitors (inhibitor solutions were serially diluted: pepstatin A, 228–1.8 μM; phenylmethylsulfonyl fluoride, 2,850–22 μM)—9, calf chymosin with pepstatin A; 10, *E. coli* chymosin with pepstatin A; 11, calf chymosin with phenylmethylsulfonyl fluoride; 12, *E. coli* chymosin with pepstatin A.

acrylamide gels (24). No new protein band comigrating with authentic prochymosin was observed. Part of the rationale in constructing pCT54 was the inclusion of two restriction sites (*Bcl* I and *Cla* I) between the Shine–Dalgarno sequence (A-A-G-G) and the ATG so that the distance between these sequence elements could be varied. As most *E. coli* mRNAs have 6–11 nucleotides between the Shine–Dalgarno sequence and the AUG (25), the distance in pCT66 (14 nucleotides) was reduced by modification at the *Cla* I site. Thus, pCT66 was cleaved with *Cla* I and incubated with nuclease S1 to remove the C-G sticky ends, and the plasmid was religated. After transformation several ampicillin-resistant colonies, from a total of 700, were selected and grown overnight in L broth. A 10-μl aliquot of each was inoculated into 1 ml of M9 medium containing glucose, casamino acids, and ampicillin (100 μg/ml) and grown for 3 hr at 37°C. Bacteria were harvested and lysed in sample buffer, and the proteins were subjected to electrophoresis on a $NaDodSO_4$/polyacrylamide gel together with a sample of purified prochymosin. The majority of clones produced a new band on induction that comigrated with the authentic prochymosin standard. The nucleotide sequence across the modified region of two of these plasmids was determined (Fig. 1*D*). pCT67 and pCT70 were shorter than pCT66 by two and three nucleotides, respectively, giving Shine–Dalgarno-to-ATG distances of 12 and 11 nucleotides. Removal of these nucleotides by nuclease S1 may promote expression of the prochymosin gene by relieving a secondary structure constraint in the mRNA (26) or, more likely, by placing the Shine–Dalgarno sequence and the ATG in the optimal configuration for formation of the initiation complex (27).

A polyacrylamide gel showing [^{35}S]methionine-labeled proteins from *E. coli* containing pCT70 or pCT54 after induction is shown in Fig. 2*A* (lanes 1 and 3). A prominent polypeptide comigrating with a calf prochymosin standard was observed in cells containing pCT70 but not in the control extract of *E. coli* containing pCT54. Evidence that this polypeptide was related to prochymosin was derived from the fact that incubation of [^{35}S]methionine-labeled proteins from *E. coli* containing pCT70 with polyclonal antiprochymosin antiserum resulted in precipitation of a protein of the same size. No protein of this size was precipitated by the antiserum from extracts of *E. coli* containing pCT54 (lanes 2 and 4). In addition, comparative two-dimensional isoelectric focusing (29) of total proteins from *E. coli* containing pCT54 or pCT70 revealed the presence of a single additional polypeptide in the pCT70 extract. This polypeptide comigrated with calf prochymosin and exhibited an apparent pI of $\approx$5.0 (data not shown).

Purification and Activation of Prochymosin. Measurement of the intensity of the Coomassie blue-stained proteins (Fig. 2*B*) by gel scanning indicated that, depending on fermentation conditions, 1–5% of total *E. coli* protein was expressed as prochymosin, equivalent to 50,000–250,000 molecules per cell. After cell lysis and centrifugation, prochymosin was detected in the cell debris by both $NaDodSO_4$/polyacrylamide gel electrophoresis and immunoprecipitation, indicating that the protein accumulates in an insoluble or aggregated form. The presence of prochymosin in the pellet fraction, however, was a useful purification step because it both concentrated the protein and separated it from the bulk of *E. coli* soluble proteins. For further purification, prochymosin was solubilized with a buffer containing 9 M urea, the urea was subsequently removed by dialysis, and the soluble enzyme was isolated by DEAE-cellulose chromatography followed by acidification. These steps removed most contaminating *E. coli* proteins and simultaneously generated chymosin (Fig. 2*B*, lanes 7, 8, and 9).

Characterization of Chymosin. The clotting activity of chymosin derived from *E. coli* containing pCT70 is compared with that of calf chymosin in Fig. 3. Both the *E. coli* and calf zymogens are inactive and require acidification for the generation of clotting activity. The enzyme is present in *E. coli* containing pCT70 but absent from cells harboring pCT54 (Fig. 3, rows 4–8). The clotting activity of both the *E. coli* and calf enzymes is sensitive to pepstatin, the specific inhibitor of aspartyl proteinases (30), and insensitive to phenylmethylsulfonyl fluoride, the inhibitor of serine proteinases (Fig. 3, rows 9–12). Furthermore, the specific activity of highly purified chymosin isolated from *E. coli* was similar to that of calf chymosin in the milk-clotting assay. It is therefore concluded that pCT70 expresses prochymosin, which can be solubilized, purified, and acid matured to produce the milk-clotting enzyme chymosin.

In conclusion, the results presented here show that biologically active enzymes expressed from eukaryotic genes can be produced in *E. coli*. Although several eukaryotic enzymes have been expressed in *E. coli* before—e.g., dihydrofolate reductase (31, 32), urokinase (33), Rous sarcoma virus *src* protein kinase (34, 35), and herpes simplex virus thymidine kinase (36, 37)—they have generally been made at low levels or in a modified form as fusion proteins or the products have not been purified. This, therefore, represents one of the few reports of the synthesis, purification, and characterization of a eukaryotic enzyme in *E. coli*. Further, this work provides a basis for the development of a process for the production of chymosin from *E. coli*. This process or one based on expression of the preprochymosin or prochymosin gene in *Saccharomyces cerevisiae* may ultimately provide an alternative source of chymosin for the cheese-making industry to compensate for the present shortage caused by a reduction in the veal calf market.

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